(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
11 January 2001 (11.01.2001)

PCT

(10) International Publication Number
WO 01/02603 A1

(51) International Patent Classification[7]: C12Q 1/68

(21) International Application Number: PCT/FI00/00587

(22) International Filing Date: 28 June 2000 (28.06.2000)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/141,286 30 June 1999 (30.06.1999) US

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(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:
— *With international search report.*
— *Before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments.*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 01/02603 A1

(54) Title: DIAGNOSTIC METHOD FOR SQUAMOUS CELL CARCINOMA

(57) Abstract: The present invention relates to diagnosis and treatment of malignant and premalignant conditions in humans. In specific the invention provides a method for characterization of squamous epithelial cells, comprising detecting from a patient sample the relative levels of the messenger RNA:s of squamous cell carcinoma antigen gene 2 (SCCA2) and squamous cell carcinoma antigen gene 1 (SCCA1), an elevated SCCA2/SCCA1 mRNA ratio indicating a malignant feature of said squamous epithelial cells.

Diagnostic method for squamous cell carcinoma

Field of the invention

The present invention relates to the diagnosis and treatment of malignant and premalignant conditions in humans. In particular, the invention relates to estimation of prognosis for planning of treatment and follow-up of patients treated for cancer, and to the diagnosis of malignant and premalignant conditions.

Background of the invention

Head and neck cancer is the sixth most common type of cancer in the world. Histopathologically, squamous cell carcinoma is the most frequent histological type of this disease. After curative treatment, about 50% of the patients develop a recurrence, and 80% of the relapses occur within the first two years of follow-up. About 50% of the patients with squamous cell carcinoma of the head and neck (SCCHN) die from the disease, with no significant improvement during the last decades.

In the management of patients with SCCHN, treatment decisions are based on the TNM classification system, although T and N status does not reliably predict the clinical outcome in individual cases. Thus, effective treatment planning would be enhanced by identification of prognostic indicators that would reflect the biological behaviour of the tumor. Although new potential prognostic markers are continuously being introduced and evaluated in SCCHN, so far none has influenced standard treatment.

Squamous cell carcinoma antigen is used as a clinical serum marker for squamous cell carcinoma of the uterine cervix, head and neck region, lung and esophagus [Kato 1992]. SCCA belongs to the serine protease inhibitor family [Suminami *et al.*, 1991] and is encoded by two highly homologous genes, SCCA1 and SCCA2 at the 18q21.3 locus [Schneider *et al.*, 1995]. SSCA1 is a potent inhibitor of lysosomal cystein proteinases [Schick *et al.*, 1998], whereas SCCA2 inhibits the lymphocyte derived serine proteinases cathepsin G and mast cell chymase [Schick *et al.*, 1997]. The neutral and acidic isoforms of

SCCA [Kato *et al.*, 1984] are encoded by the SCCA1 and SCCA2 genes, respectively [Schneider *et al.*, 1995]. The proportion of the acidic isoform is increased in malignant epithelium and it is mainly this form that is secreted into circulation in epithelial malignancies [Kato, 1992].

Summary of the invention

The relative levels of SCCA2 and SCCA1 mRNA in malignant squamous epithelial cells reflect the biological behavior of the tumor. By measuring the SCCA2/SCCA1 mRNA ratio in a sample containing malignant or suspectedly malignant squamous epithelial cells and comparing the ratio obtained with a method-specific cut-off value or scale, the risk of the patient to develop recurrent disease after treatment can be estimated. This method provides prognostic information for planning of treatment and follow-up of patients treated for squamous cell carcinoma. The method can also be used for diagnostic characterization of malignant or suspectedly malignant squamous epithelial lesions and could provide a potentially useful tool for molecular grading of squamous cell carcinomas of various sites.

Detailed description of the invention

We used a new RT-PCR-based technique to accurately determine the ratio of the mRNA levels of the SCCA genes 1 and 2 in microscopic frozen sections from squamous cell carcinoma tumor and normal epithelium tissue specimens from the head and neck region. For the purposes of this invention the head and neck region includes the oral cavity, the nasal cavity, the paranasal sinuses, the pharynx, the larynx and the salivary glands. Further, a tumor or normal epithelium specimen can also be obtained from the esophagus, the trachea, the lung or the uterine cervix. As the SCCA genes are tissue-specifically expressed in squamous epithelial cells, it is possible to accurately quantitate the tumor derived relative mRNA levels of these two genes in microscopic sections containing a mixture of cell types. An internal standard in the PCR reaction ensures that a sufficient amount of mRNA template has been present, and thus samples can be assayed without prior knowledge of the exact amount of analysed cells. Thus, the ratio of the two closely related mRNAs occurring in the same cells are measured. The SCCA1 and SCCA2 derived PCR products are separ-

ately detected by solid-phase minisequencing on the basis of a mismatch at a single nucleotide position. The SCCA2/SCCA1 mRNA ratio in the original sample is determined by extrapolating the PCR product ratio against a standard curve of PCR calibrators, and compared with a method-specific cut-off level.

With the cut-off level used, the assay was capable of predicting recurrence of malignant disease in 9 of 10 patients with primary squamous cell carcinoma tumors in the head and neck region. The relative risk of a patient with a primary tumor displaying an elevated SCCA2/SCCA1 mRNA ratio, to develope a recurrence, was 9.6.

The SCCA2/SCCA1 mRNA ratio was a prognostic factor independent of stage in this material. In the patients presenting with a stage I-II disease, there were no recurrences among the patients displaying SCCA2/SCCA1 mRNA ratios below the cut-off level in the primary tumor. In the patients presenting with a stage III-IV disease, there was one recurrence among the patients displaying an SCCA2/SCCA1 mRNA ratio below the cut-off level in the primary tumor.

In conclusion, this novel technique is a potential tool for grading of squamous cell carcinomas of the head and neck region and of other sites such as the uterine cervix, lung, trachea and esophagus. The SCCA2/SCCA1 mRNA ratio is a predictor of recurrence after treatment in SCCHN and can possibly also be used for diagnostic characterization of premalignant lesions.

In general, relative levels of messenger ribonucleic acid (mRNA) of cell-type specifically expressed genes, measured in samples of tissue or body fluids, containing malignant or suspectedly malignant cells, can be used for estimation of prognosis of patients treated for cancer and for diagnosis of malignant and premalignant conditions arising from this type of cells.

The RT-PCR based technique used, including the minisequencing assay used for quantitation of the PCR products, enables accurate quantitative measurement of the relative mRNA levels of two highly homologous genes or splicing variants in biological samples

consisting of minute amounts of target cells. However, the determination of the mRNA ratios according to the present invention should not be considered restricted to the RT-PCR or minisequencing techniques as used in the present experiment, but any other suitable method for measuring relative mRNA levels can be used.

The fact that pathological specimens routinely taken from cancer patients can directly be used for a determination at the nucleic acid level, is one of the advantageous features of the present technique. It should also be appreciated that with this technique a decision on the treatment of the disease can be made on the basis of the mRNA expression pattern of the primary tumor.

Brief description of the drawings

Fig. 1. Kaplan-Meier curves displaying recurrence-free survival over time in patients presenting with primary tumors displaying high vs. low SCCA2/SCCA1 mRNA ratios.

Fig. 2a. Kaplan-Meier curves displaying recurrence-free survival over time in patients presenting with T1 or T2 primary tumors displaying high vs. low SCCA2/SCCA1 mRNA ratios.

Fig. 2b. Kaplan-Meier curves displaying recurrence-free survival over time in patients presenting with T3 or T4 primary tumors diplaying high vs. low SCCA2/SCCA1 mRNA ratios.

Fig. 3. Scatter plot displaying distribution and means of SCCA2/SCCA1 mRNA ratios measured in normal epithelium samples, primary tumors from patients eventually developing recurrent disease, primary tumors from patients not developing recurrent disease during follow-up, and recurrent tumors.

Materials and methods

The ratio of SCCA2/SCCA1 mRNA in patient and control samples was determined by a novel RT-PCR based technique (see below) [Stenman *et al.*, 1999].

In brief: Total RNA was extracted and reverse transcribed using a common specific primer for the SCCA1 and SCCA2 transcripts. The cDNA samples templates were coamplified in a nested PCR with one thousand copies of an internal standard. Following amplification, the PCR products were separately detected by solid-phase minisequencing on the basis of a sequence difference at a single nucleotide position. The original mRNA ratio was estimated by extrapolating the PCR product ratio against a standard curve of PCR calibrators.

Patient samples

Tumor samples from 32 patients treated with curative intent for squamous cell carcinoma of the head and neck region at the Department of Otorhinolaryngology, Turku University Central Hospital between the years 1993 and 1999 were included in the study in accordance with the Helsinki declaration. All patients underwent radiation therapy, 28 patients underwent surgery. Tumor samples were obtained as biopsies before treatment or at surgery and were stored at –40°C until assayed.

The follow-up program included clinical evaluation and, when indicated, endoscopic examination every third month for two years, and thereafter during one to three annual follow-up visits. Clinical data on recurrence and overall survival was retrieved from clinical records.

Sample preparation

Tumor (n=32) and control (n=19) samples consisted of five 10 µm microscopic frozen sections of squamous cell carcinoma tumor specimens and normal epithelium surgically removed from the head and neck region. The area of the sections varied from approximately 1 to 50 mm^2. The frozen sections were added directly to 750 µl of RLT lysis buffer (Qiagen, Hilden, Germany) containing guanidine isothiocyanate and stored at -70°C. The

proportion of the different cell types in the sample was estimated from an adjacent toluidine blue stained section by an experienced pathologist.

Samples were homogenized by centrifuging the lysate through a QIAshredder spin column (QIAgen). Total RNA was extracted using RNeasy Mini spin columns (QIAgen) and treated with 2 U of RQ1 RNase-free DNase (Promega, Madison, WI) at 37°C for 30 min according to the manufacturer's instructions. One fifth of the total RNA was reverse transcribed with Superscript II® reverse transcriptase (Life Technologies, Rockville, MD) according to the instructions, using the common outer antisense PCR primer.

Wildtype templates and internal standard

The SCCA1 and SCCA2 templates differ within the PCR amplicon at positions 206, 211 and 212 of the SCCA2 cDNA sequence (Genebank, accession number: U19557). An internal standard was constructed by generating an additional A to T mismatch at position 206 of the SCCA2 cDNA using modified primers and PCR. The generated sequence was amplified with the outer PCR primers, cloned into the pCR® II vector (Invitrogen, Carlsbad, CA) and sequenced from both ends on an ABI 310 genetic analyzer (Perkin-Elmer, Norwalk, CT) using the ABI Prism dRhodamine terminator cycle sequencing kit (Perkin-Elmer). One thousand copies of the nonlinearized vector containing the modified SCCA2 sequence was included in the PCR reactions as an internal standard.

PCR and minisequencing primers

The outer PCR primers were: 5'-TTCTATTCCCCTATCAGCATC-3' (sense), 5'-TTGCA-GCTTTTTCTGTGGT-3' (antisense) and the inner were: 5'-biotin-GCATCACATCAG-CATTAGG-3' (sense) and 5'-GCTTTTTCTGTGGTGTTCTC-3' (antisense). The PCR amplicon spans the exon 2-3 junction of the SCCA1 and SCCA2 genes. The minisequencing primers: 5'-GTGAAGAACCTTCTTAAT-3' (SCCA1) and: 5'-GTGAAGAACCTTG-CTAAT-3' (SCCA2 and the internal standard) both bind symmetrically on the exon 2-3 junction.

Amplification and detection of PCR products

One µl of the cDNA was transferred to a 40 µl PCR reaction containing 0.25 mM of each dNTP, 20 pmol of the outer sense and antisense primers, 1.6 U of Dynazyme II polymerase (Finnzymes, Espoo, Finland) and 1 x PCR buffer (10 mM Tris-HCl, pH 8.8, 1.5 mM $MgCl_2$, 50 mM KCl, 1 ml/l Triton X-100) supplied with the enzyme. The internal standard was added to the master mix to give one thousand copies per PCR reaction. Thirty cycles of amplification at 94°C for 30 s, 57°C decreasing by 0.1°C for each cycle for 1 min and 72°C for 30 s was performed on a Geneamp 2400 cycler (Perkin-Elmer). Two µl of the PCR product was transferred to a 100 µl nested PCR reaction containing 20 pmol of the 5'-biotinylated inner sense primer and 100 pmol of the inner antisense primer and 4 U of Dynazyme II polymerase. Thirty additional amplification cycles were performed at 94°C for 1 min, 55°C for 1 min and 72°C for 30 s. Following amplification, the biotinylated sense strand of the nested PCR product was captured on a Scintistrip® streptavidin coated scintillating microtitration plate (EG&G Wallac, Turku, Finland). The three end point PCR products were separately labeled with ^3H-labeled nucleotides by solid-phase minisequencing and the incorporated radioactivity was measured in a beta counter and expressed as counts per minute (CPM) [Syvänen *et al.*, 1990; Suomalainen and Syvänen, 1998; Ihalainen *et al.*, 1994].

Results

In the control group of normal epithelium samples (n=19) the SCCA2/SCCA1 mRNA ratios where in the range 0.001-0.418 (median 0.061). The upper 95% and 99% confidence intervals for the median were 0.162 and 0.233, respectively. A cut-off level for SCCA2/SCCA1 mRNA ratios considered elevated was set at 0.200.

The SCCA2/SCCA1 mRNA ratio was elevated above the cut-off level in 9 of 10 patients developing recurrent disease after a period of disease free follow-up after treatment. In the group of patients not developing recurrent disease during follow-up, the SCCA2/SCCA1 mRNA ratio was elevated above the cut-off level in 5 of 13 patients. The relative risk of developing a recurrence after treatment for a patient with a primary tumor displaying an

SCCA2/SCCA1 mRNA ratio elevated above the cut-off level was 9.6 [95% CI 1.2-80, Cox regression analysis adjusted for high (T3-4) and low (T1-2) stage].

Nine of 14 patients with a primary tumor displaying an elevated SCCA2/SCCA1 mRNA ratio experienced a recurrence of disease within two years of follow-up. One of nine patients with a primary tumor displaying an SCCA2/SCCA1 mRNA ratio below the cut-off level had a recurrence within the follow-up period. The SCCA2/SCCA1 mRNA ratio in primary tumors was a predictor of recurrent disease, using a cut-off level of 0.200 ($p=0.030$, logrank test for equality of recurrence-free survival distributions) (Fig. 1).

The SCCA2/SCCA1 mRNA ratio predicted recurrence independently of stage ($p=0.210$, logrank test for equality of recurrence-free survival distributions, adjusted for low (T1-2) and high (T3-4) stage. In the group of patients presenting with stage I or II tumors, 5 of 9 patients with an elevated SCCA2/SCCA1 mRNA ratio developed recurrent disease within 2 years of follow-up. In this group none of the 3 patients with an SCCA2/SCCA1 mRNA ratio below the cut-off level developed recurrent disease during the follow-up period (Fig. 2a.). In the group of patients presenting with stage III or IV tumors 4 of 5 patients with an elevated SCCA2/SCCA1 mRNA ratio developed recurrent disease within 2 years of follow-up. In this group 1 of the 6 patients with an SCCA2/SCCA1 mRNA ratio below the cut-off level developed recurrent disease during the follow-up period (Fig. 2b.).

In the group of primary tumors eventually developing into recurrent disease ($n=10$) and primary tumors not developing into recurrent disease during follow-up after treatment ($n=13$) the SCCA2/SCCA1 mRNA ratios where in the ranges 0.173-0.649 (median 0.253) and 0.013-0.753 (median 0.186), respectively. In the group of recurrent tumors ($n=13$) the SCCA2/SCCA1 mRNA ratios where in the range 0.037-0.789 (median 0.380). The SCCA2/SCCA1 mRNA ratios where significantly higher in the groups of primary ($p = 0.016$, and $p < 0.001$ respectively) and recurrent tumors ($p < 0.001$) compared to the normal epithelium samples ($n=19$) (Mann-Whitney U-test) (Fig. 3.).

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Claims

1. A method for characterization of squamous epithelial cells, comprising measuring from a patient sample the relative levels of the messenger RNA:s of squamous cell carcinoma antigen gene 2 (SCCA2) and squamous cell carcinoma antigen gene 1 (SCCA1); the SCCA2/SCCA1 mRNA ratio reflecting the biological behaviour of said squamous epithelial cells.

2. A diagnostic method for characterization of malignant or suspectedly malignant squamous epithelial cells from a patient, comprising the steps of
- obtaining a sample of cells, tissue, or body fluid, containing malignant or suspectedly malignant squamous epithelial cells of said patient,
- determining the relative levels of the messenger RNA:s of squamous cell carcinoma antigen gene 2 (SCCA2) and squamous cell carcinoma antigen gene 1 (SCCA1), and
- comparing the ratio obtained with a method-specific cut-off value or scale;
an elevated SCCA2/SCCA1 mRNA ratio indicating a malignant feature of said squamous epithelial cells.

3. A prognostic method for predicting recurrence of squamous cell carcinoma in a patient, comprising the steps of
- obtaining a sample of cells, tissue, or body fluid, containing malignant or suspectedly malignant squamous epithelial cells of said patient,
- determining the relative levels of the messenger RNA:s of squamous cell carcinoma antigen gene 2 (SCCA2) and squamous cell carcinoma antigen gene 1 (SCCA1), and
- comparing the ratio obtained with a method-specific cut-off value or scale;
an elevated SCCA2/SCCA1 mRNA ratio indicating an increased risk of recurrence after treatment.

4. A prognostic method for planning of treatment and follow-up of patients treated for squamous cell carcinoma, comprising the steps of
- obtaining a sample of cells, tissue, or body fluid, containing malignant or suspectedly malignant squamous epithelial cells of said patient,

- determining the relative levels of the messenger RNA:s of squamous cell carcinoma antigen gene 2 (SCCA2) and squamous cell carcinoma antigen gene 1 (SCCA1), and
- comparing the ratio obtained with a method-specific cut-off value or scale;
an elevated SCCA2/SCCA1 mRNA ratio indicating a need of aggressive treatment and intensive follow-up.

5. The method according to any one of claims 1-4, to determine the relative levels of the messenger RNA:s of squamous cell carcinoma antigen gene 2 (SCCA2) and squamous cell carcinoma antigen gene 1 (SCCA1), wherein the sample contains squamous epithelial cells originating from the head and neck region, including the oral cavity, the nasal cavity, the paranasal sinuses, the pharynx, the larynx and the salivary glands.

6. The method according to any one of claims 1-4, to determine the relative levels of the messenger RNA:s of squamous cell carcinoma antigen gene 2 (SCCA2) and squamous cell carcinoma antigen gene 1 (SCCA1), wherein the sample contains squamous epithelial cells originating from the esophagus.

7. The method according to any one of claims 1-4, to determine the relative levels of the messenger RNA:s of squamous cell carcinoma antigen gene 2 (SCCA2) and squamous cell carcinoma antigen gene 1 (SCCA1), wherein the sample contains squamous epithelial cells originating from the trachea or the lung.

8. The method according to any one of claims 1-4, to determine the relative levels of the messenger RNA:s of squamous cell carcinoma antigen gene 2 (SCCA2) and squamous cell carcinoma antigen gene 1 (SCCA1), wherein the sample contains squamous epithelial cells originating from the uterine cervix.

Figure 1

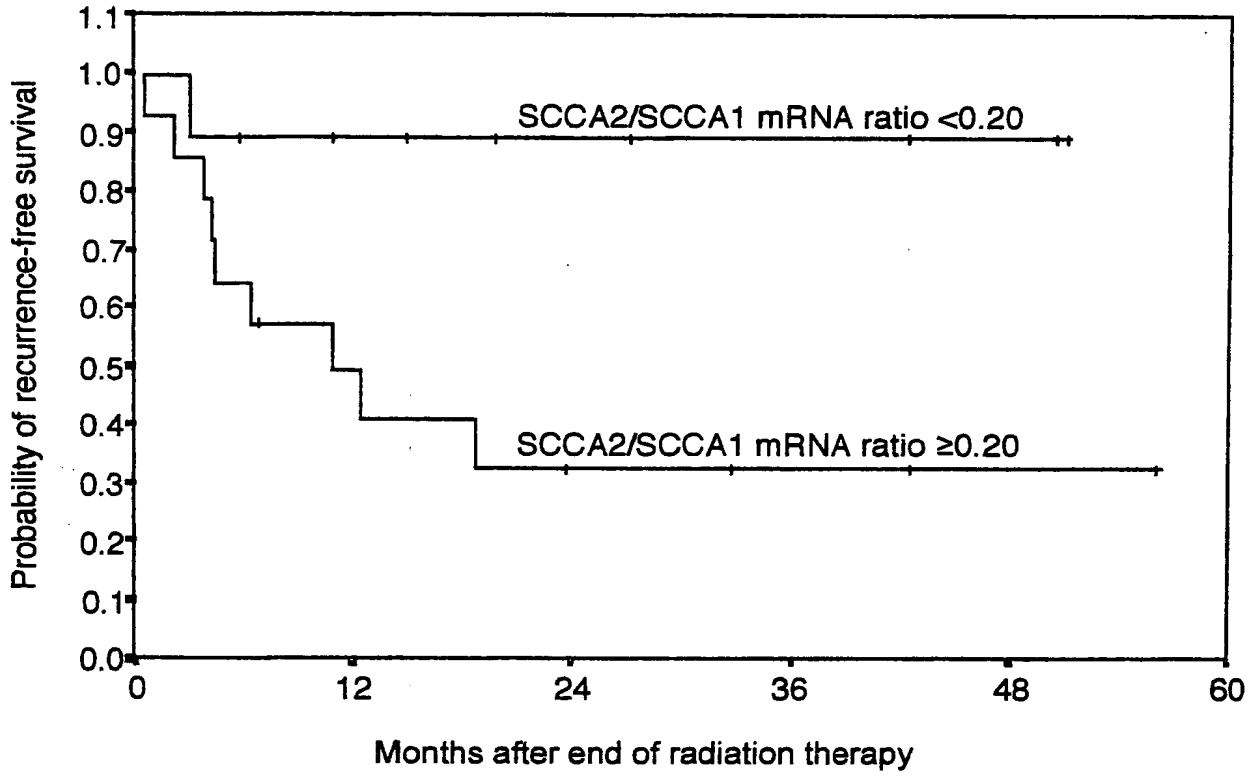

1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
Probability of recurrence-free survival
SCCA2/SCCA1 mRNA ratio <0.20
SCCA2/SCCA1 mRNA ratio ≥0.20
0
12
24
36
48
60
Months after end of radiation therapy

Figure 2a

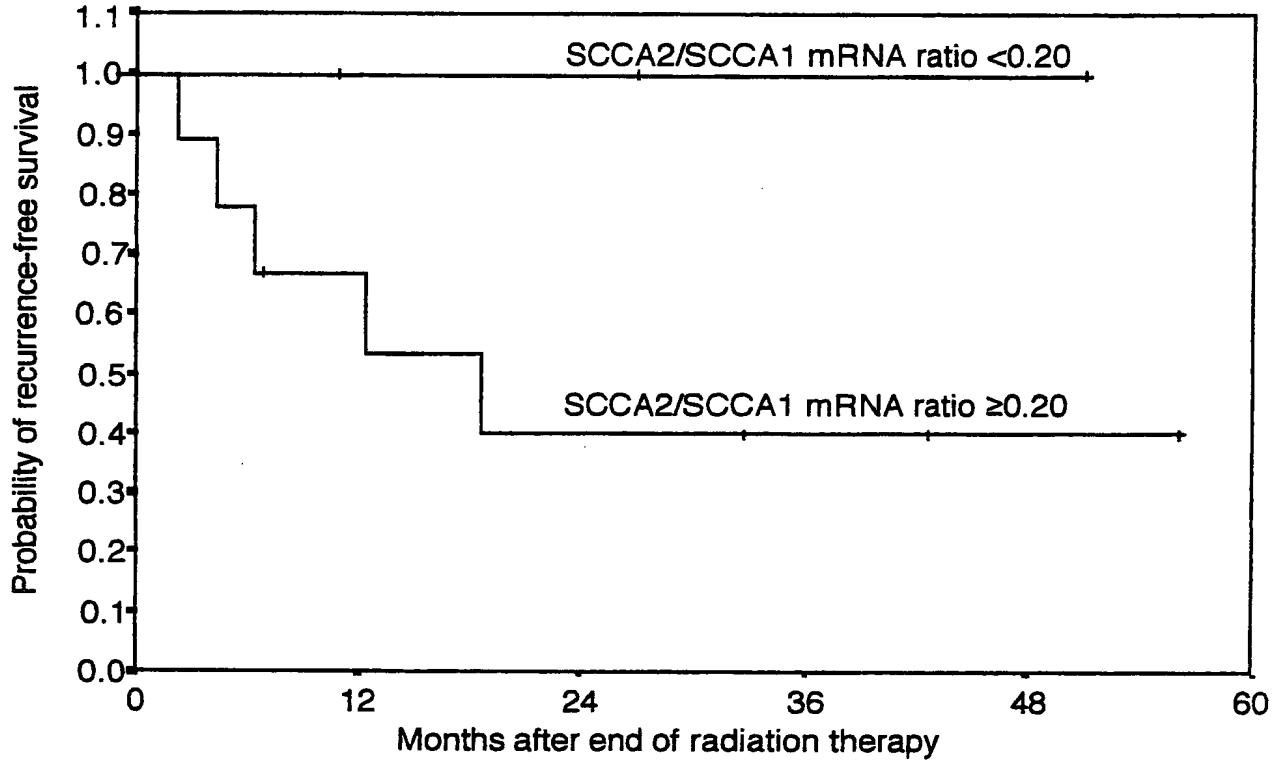


Figure 2b

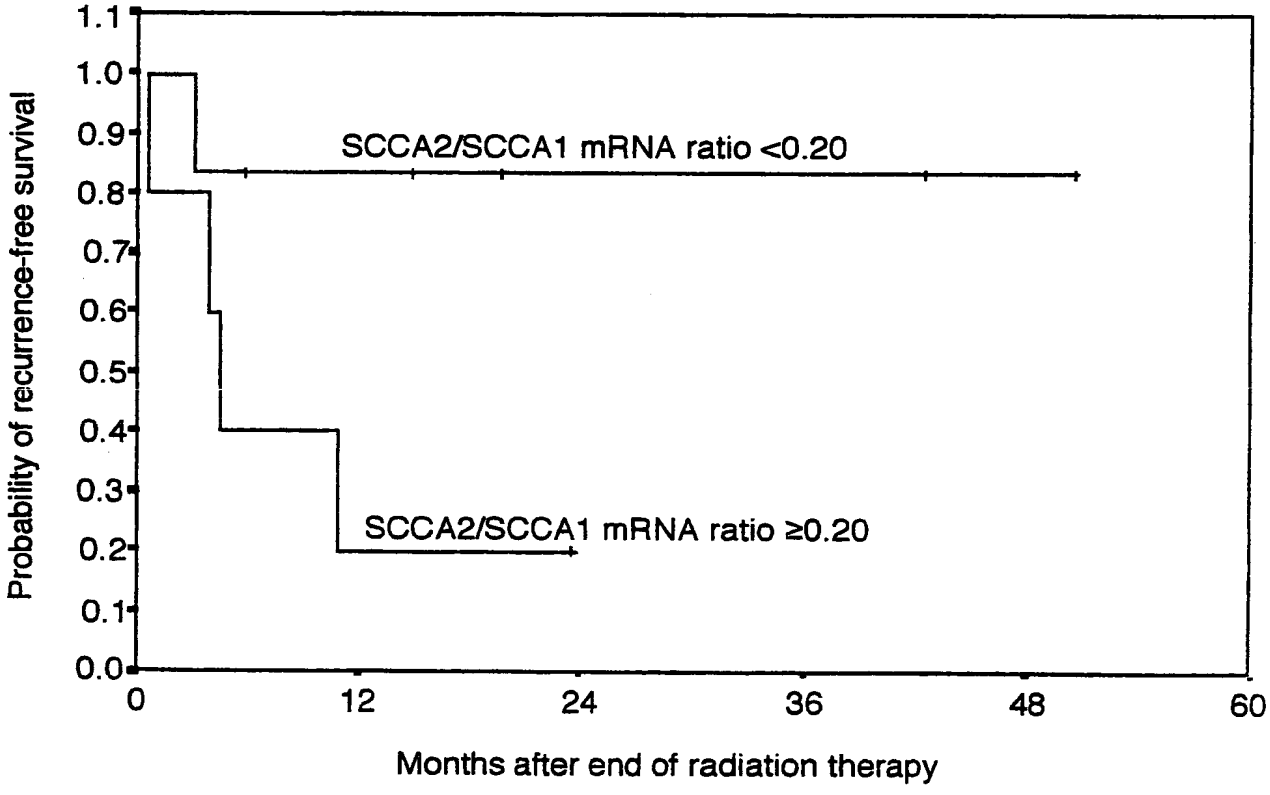
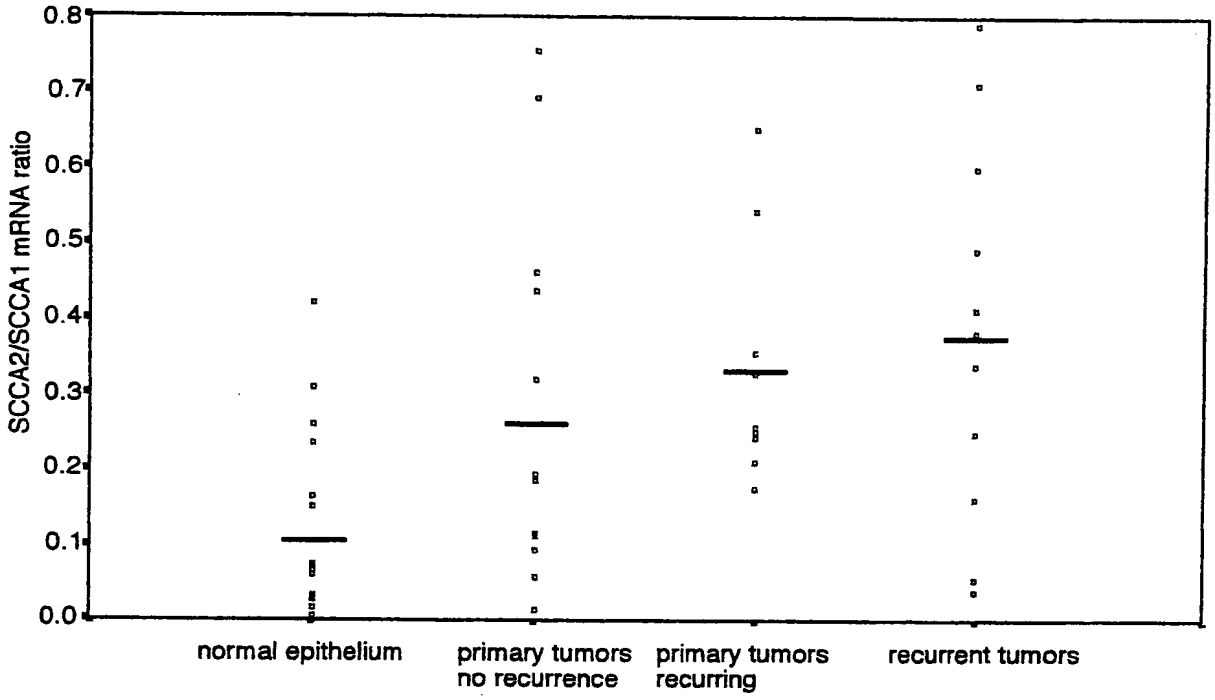


Figure 3


SCCA2/SCCA1 mRNA ratio
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
normal epithelium
primary tumors no recurrence
primary tumors recurring
recurrent tumors

INTERNATIONAL SEARCH REPORT

International application No.
PCT/FI 00/00587

A. CLASSIFICATION OF SUBJECT MATTER

IPC7: C12Q 1/68
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC7: C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	Nature Biotechnology, Volume 17, 1999, Jakob Stenman et al, "Accurate determination of relative messenger RNA levels by RT-PCR" page 720 - page 722 --	1-8
Y	JP 10215881 A (SHISEIDO CO LTD), 18 August 1998 (18.08.98), see technical problem (0007), last sentence (0019) and (0020), effect of the invention (0044), and automatic translation from http://www.ipdl.jpo-miti.go.jp/homepg-e.ipdl --	1-8

[X] Further documents are listed in the continuation of Box C. [X] See patent family annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
25 October 2000	03 -11- 2000
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INTERNATIONAL SEARCH REPORT

International application No.
PCT/FI 00/00587

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Gann, Volume 75, May 1984, Hiroshi Kato et al, "Heterogeneity of a tumor antigen TA-4of squamous cell carcinoma in relation to its appearance in the circulation", page 433 - page 435, see abstract --	1-8
A	Int. J. Cancer (Pred. Oncol.), Volume 74, 1997, Jakob Stenman et al, "Detection of squamous-cell carcinoma antigen-expressing tumour cells in blood by reverse transcriptase-polymerase chain reaction in cancer of the uterine cervix", page 75 - page 80, page 78, column 1, lines 3-24 -- --------	1-8

INTERNATIONAL SEARCH REPORT

Information on patent family members

03/10/00

International application No.
PCT/FI 00/00587

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
JP 10215881 A	18/08/98	NONE	